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Babesia spp. Identified by PCR in Ticks Collected from Domestic and Wild Ruminants in Southern Switzerland

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Concurrent infections with vector-borne pathogens affected a cattle herd in Switzerland, and one of the pathogens was identified as *Babesia bigemina*, which had never been observed in this country before. Therefore, a survey of the occurrence of ruminant *Babesia* spp. and their tick vectors in Switzerland was conducted. A total of 2,017 ticks were collected from sheep, goats, cattle, and wild ruminants (deer, roe deer, and chamois) in southern parts of Switzerland and identified morphologically. The vast majority of the ticks (99.2%) were *Ixodes ricinus*, but 14 ticks from sheep and goats were identified as *Dermacentor marginatus* and two ticks from wild ruminants were identified as *Hemaphysalis punctata*. PCR analyses of 700 ticks revealed the presence of *Babesia divergens* ($n = 6$), *Babesia* sp. genotype EU1 ($n = 14$), and *B. major* ($n = 2$), whose suggested occurrence was confirmed in this study by molecular analysis, and the presence of novel *Babesia* sp. genotype CH1 ($n = 4$), which is closely related to *B. odocoilei* and to *Babesia* sp. genotype RD61 reported from North America. The identification of *B. divergens* and *B. major* in ticks collected from wild ruminants cast doubt on the postulated strict host specificity of these bovine *Babesia* species. Furthermore, the zoonotic *Babesia* sp. genotype EU1 was detected in ticks collected from domestic animals but was obtained predominantly from ticks collected from wild ruminants. More than one tick containing DNA of different *Babesia* spp. were collected from two red deer. Hence, the role of these game animals as reservoir hosts of *Babesia* spp. seems to be important but requires further investigation.

A fatal disease outbreak of anaplasmosis affected a cattle herd in Chur, the capital city of the Grisons canton in eastern Switzerland (18). Together with *Anaplasma* spp. and *Mycoplasma* sp., concurrent infections with piroplasms were detected, which were identified by PCR and sequencing as *Babesia bigemina*, a large *Babesia* species, and *Theileria* belonging to the *Theileria buffeli* complex (H. Hilpertshauser, P. Deplazes, M. L. Meli, R. Hofmann-Lehmann, H. Lutz, and A. Mathis, submitted for publication). Both these piroplasm species are common bovine parasites in warmer climates in Europe but had never been observed in Switzerland before.

The occurrence of a large bovine *Babesia* sp. in Switzerland has been reported only once before. An infection with *B. major*, which is considered a species with low virulence, was suggested based on diagnosis by light microscopy of blood smears of a cow (7). In contrast, *B. divergens*, a small *Babesia* species, is sporadically observed as an organism causing clinical infections in several places in Switzerland, especially in the southern and western parts of the country (16).

The transmission of *Babesia* is thought to be strictly associated with ticks (15). By far the most common tick species in Switzerland is *Ixodes ricinus* (3), a proven vector of *B. divergens*, and DNA of this parasite has recently been detected by PCR in ticks collected in southern and western regions of Switzerland (9). Additional ticks that are found in Switzerland include other species in the genus *Ixodes*, *Dermacentor marginatus*, *Dermacentor reticulatus*, *Hemaphysalis punctata*, and *Rhipicephalus sanguineus* (3). The presence of *Rhipicephalus bursa* and *Hemaphysalis sulcata* on goats and cattle in southern parts of Switzerland was reported once (2), but these two species were not found in several subsequent investigations (3). *H. punctata* is a proven vector of *B. major* (27). However, none of the indigenous tick species of Switzerland is considered a vector of *B. bigemina*.

The aim of this study was to investigate further the epidemiology of *B. bigemina* in Switzerland. A survey was conducted of *Babesia* spp. in ticks collected from domestic ruminants and also from wild ruminants in Ticino and Poschiavo, two regions in southern Switzerland (south of the Alps).

(This work was part of the veterinary thesis of Heidi Hilpertshauser.)

MATERIALS AND METHODS

Collection of ticks. In spring and/or summer 2004, ticks were collected from animals in two distinct southern alpine regions (Poschiavo Valley in Grisons canton and in Ticino canton) bordering northern Italy. A total of 916 ticks were collected from cattle, goats, and sheep from 14 farms in Poschiavo, and 1,101 ticks were collected from shot deer, roe deer, and chamois from the two areas in autumn 2004. Ticks were stored in 70% ethanol at 4°C. The species, stage, and gender of each tick were determined with a stereo microscope using the key of the University of Neuchâtel (11).

DNA extraction. Ticks were processed individually or pooled in groups. Generally, three ticks from each animal were examined in one pooled sample, and, if the sample was positive for a *Babesia* sp., additional ticks from the same animal were analyzed individually. First, each tick was washed three times in sterile phosphate-buffered saline and then stored at −20°C. The frozen ticks were cut into pieces with ethanol-flamed scissors in 1.5-ml Eppendorf tubes. DNA from fully engorged females was isolated from only the apical part, 400 μl of 25% Chelex (Bio-Rad) was added, and the samples were subjected to three cycles of freezing and thawing. Digestion with proteinase K (200 μg/ml; Roche, Mannheim, Germany) was performed by incubation at 56°C overnight. After centrifugation at maximum speed for 10 min, the supernatant was transferred into a

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TABLE 1. Features of PCR primers used in this study and reaction conditions

Specificity	Locus	Sequence (5′–3′)	Fragment size (bp)	Annealing temp (°C)	Extension time (s)	Reference
Babesia spp.[a]	Part of the 18S rRNA gene	GTTTCTGMCCCATCAGCTTGAC CAAGACAAAAGTCTGCTTGAAAC	422–440	61	45	This study
B. divergens	Part of the 18S rRNA gene	GTTTCTGMCCCATCAGCTTGAC CAATATTAACACCACGCAAAAATTC	353	61	45	This study
Babesia sp. genotype EU1	Part of the 18S rRNA gene	GTTTCTGMCCCATCAGCTTGAC AGACAAGAGTCAATAACTCGATAAC	362	61	45	This study
Ticks	Part of the mitochondrial 16S rRNA gene	CCGGTCTGAACTCAGATCAAG CAATGATTWTTTAAATTGSTGTGG	448–457	58	45	4 (modified)

[a] Bovine *Babesia* spp. (*B. divergens*, *B. bigemina*, *B. major*), *Babesia* sp. genotype EU1, *B. canis*, *B. odocoilei*, *B. ovata*, *B. motasi*, and *B. crassa*.

fresh tube. DNA was isolated by phenol-chloroform extraction and ethanol precipitation. DNA pellets were washed once with ice-cold 70% ethanol, air dried, and resuspended in 200 μl of Tris-HCl (10 mM, pH 8.4).

PCR. Primers and cycling conditions used in this study are listed in Table 1. Primers with different specificities for *Babesia* spp. were deduced from the aligned GenBank entries for species of the genera *Babesia* and *Theileria*, as well as unspecified members of the Piroplasmida (see Fig. 1 for accession numbers), and the specificities were confirmed by BLAST searches. Each 100-μl PCR mixture contained 5 μl extracted DNA, buffer (50 mM KCl, 20 mM Tris-HCl [pH 8.4], 2.5 mM $MgCl_2$, 0.5% Tween 20), each deoxynucleoside triphosphate at a concentration of 0.2 mM (with dUTP replacing dTTP) (Sigma-Aldrich, Buchs, Switzerland), each primer (Table 1) at a concentration of 1 μM, and 0.5 U uracil DNA glycosylase (Sigma-Aldrich, Buchs, Switzerland); the uracil DNA glycosylase was used to control for PCR carryover contamination (24). An initial step at 37°C for 10 min was performed in an automatic thermal cycler. After 10 min of heat inactivation of the uracil DNA glycosylase, 2.5 U *Taq* polymerase (Sigma-Aldrich, Buchs, Switzerland) was added for a hot start, followed by 45 cycles of denaturation for 30 s at 94°C, annealing for 30 s at the temperatures shown in Table 1, and extension for the times shown in Table 1 at 72°C and then a final incubation for 10 min at 72°C. Amplicons were visualized with ethidium bromide after electrophoresis in 1.5% agarose gels.

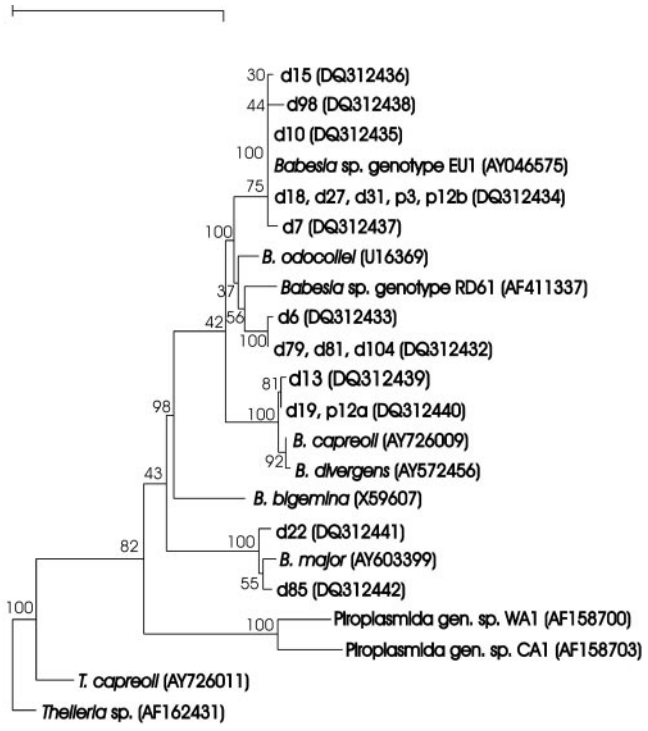

FIG. 1. Dendrogram of partial 18S rRNA gene sequences of *Babesia* spp. detected in ticks by PCR/sequencing and of selected piroplasmids (neighbor-joining analysis). The numbers at the nodes are bootstrap values based on 100 replicates. Bar = 0.1 mutation per site. Isolate designations (d, isolate from ticks collected from deer in Ticino; p, isolate from sheep and goats in the Poschiavo Valley) and GenBank accession numbers (in parentheses) are given. Isolates d86 (*B. divergens*), pd14 (isolate from ticks collected from deer from the Poschiavo Valley), and d49 (*Babesia* sp. genotype EU1) were omitted because of ambiguous sequence positions.

DNA sequencing was done by a private company (Microsynth, Balgach, Switzerland) either directly with the amplicons after DNA purification with a QIAquick PCR purification kit (QIAGEN, Hilden, Germany) or after another PCR with deoxynucleoside triphosphates containing dTTP and cloning into a TOPO TA cloning kit (Invitrogen, Carlsbad, Calif.) used according to the manufacturer's instructions.

Sequences were subjected to BLAST searches in GenBank, and a neighbor-joining tree was constructed in combination with the bootstrap method (26), using the aligned sequences (MultiAlin) (10).

To check whether amplifiable DNA was extracted, PCR (40 cycles) employing tick-specific primers was performed (Table 1).

Nucleotide sequence accession numbers. The accession numbers for 11 sequences determined in this study that have been deposited in the GenBank database are shown in Fig. 1.

RESULTS

Tick species. The vast majority of the 2,017 ticks collected both from domesticated ruminants and from wild ruminants were identified as *I. ricinus* (n = 2,001) (Table 2). Collection from sheep and goats in the spring and summer also yielded a few nymphs and larvae of *I. ricinus*. The ticks collected from sheep and goats in the spring included 14 *D. marginatus* adults. Two adult *H. punctata* ticks were collected from a roe deer and a red deer in Ticino.

Sequence analysis of *Babesia* DNA detected in ticks. First, PCR with primers detecting a broad range of *Babesia* spp. (Table 1) was performed. Hence, for 700 ticks analyzed, DNA of *Babesia* spp. was detected in 26 samples, and 21 amplicons were sequenced, revealing four different species or genotypes (Fig. 1). Isolates d13, d19, d86, and p12a clustered together with *B. divergens*. The sequences of 11 isolates (d7, d10, d15, d18, d27, d31, d49, d98, p3, p12b, and pd14) formed a group with *Babesia* sp. genotype EU1 (accession no. AY046575) amplified from blood samples from two human patients (17). Isolates d22 and d85 showed the highest level of identity (98%) with the single sequence of *B. major* that is available for this

TABLE 2. Identification of ticks collected in southern Switzerland from animals in spring, summer, and autumn 2004 and *Babesia* spp. detected in ticks by PCR and sequencing or specific PCR[a]

Location	Hosts	Ticks			*Babesia* spp. (no. of isolates)
		Species	Stage	Total no./no. tested for *Babesia* spp.	
Poschiavo Valley	Sheep, goat, cattle	*Ixodes ricinus*	Adults	875/111	*B. divergens* (1), *Babesia* sp. genotype EU1 (2)
			Nymphs	26/0	
			Larvae	1/0	
		Dermacentor marginatus	Adults	14/14	
	Deer, roe deer, chamois	*Ixodes ricinus*	Adults	32/32	*Babesia* sp. genotype EU1 (1)
Ticino	Deer, roe deer, chamois	*Ixodes ricinus*	Adults	1,067/541	*B. divergens* (5), *Babesia* sp. genotype EU1 (10), *B. major* (2), *Babesia* sp. genotype CH1 (4)
		Hemaphysalis punctata	Adults	2/2	*Babesia* sp. genotype EU1 (1)

[a] Reactions were performed with primers specific for *B. divergens* or *Babesia* sp. genotype EU1 (see Table 1).

part of the 18S rRNA gene. A novel sequence more distantly related to known sequences and therefore designated *Babesia* sp. genotype CH1 (d6, d79, d81, and d104) was most closely related to sequences reported from North America, namely sequences of *B. odocoilei* and *Babesia* sp. (RD61).

DNA of *Babesia* spp. was detected in 26 samples employing PCR with broad specificity. PCR using specific primers revealed the presence of *B. divergens* and *Babesia* sp. genotype EU1 in two and three samples, respectively. Twenty-one amplicons were sequenced, revealing four different species or genotypes; these amplicons included 4 *B. divergens* amplicons, 11 *Babesia* sp. genotype EU1 amplicons, 4 *Babesia* sp. CH1 amplicons, and 2 *B. major* amplicons.

Detection of DNA of *Babesia* spp. in ticks. Six *I. ricinus* females, one collected from a goat in Poschiavo, one collected from a chamois, and four collected from three red deer in Ticino, were infected with *B. divergens* (Table 3). *Babesia* sp. genotype EU1 was found not only in *I. ricinus* females from domesticated and wild ruminants in both Poschiavo and Ticino but also in two *I. ricinus* males and one *H. punctata* female. Analysis of *I. ricinus* collected from red deer revealed the presence of *B. major* in two female ticks. The novel sequence designated *Babesia* sp. genotype CH1 was obtained from four *I. ricinus* females picked from red deer in Ticino. More than one of the ticks collected from four red deer shot in Ticino contained DNA of *Babesia* spp. (Table 3). One male and one female *I. ricinus* from red deer 17 tested positive for *Babesia* sp. genotype EU1. Two *B. divergens*-positive ticks from red deer 39 were sampled. Both *B. major*-positive sample d22 and one sample (d75) which was PCR positive specifically for *Babesia* sp. genotype EU1 were obtained from ticks from another red deer (red deer 48). Finally, all 16 ticks recovered from red deer 30 were analyzed by PCR individually or in pools (a total of 13 samples), which revealed the presence of *B. divergens* and *B. major* in one sample each, the presence of *Babesia* sp. genotype EU1 in two samples, and the presence of the novel *Babesia* sp. genotype CH1 in three samples. All sheep and goats in the Poschiavo Valley from which the *Babesia*-positive ticks were collected were on the same farm.

Control of the efficiency of DNA extraction. A total of 74 DNA samples were tested with the primers for amplification of tick DNA to check DNA extraction. A fragment of the expected size (approximately 450 bp) was obtained from 60 sam-

TABLE 3. *Babesia* spp. identified by PCR based on DNA isolated from ticks collected from domestic and wild ungulates from two locations

Location	Animal(s)	Isolates[a]			
		B. divergens ($n = 6$)	*Babesia* sp. genotype EU1 ($n = 14$)	*B. major* ($n = 2$)	*Babesia* sp. strain CH1 ($n = 4$)
Poschiavo Valley	Sheep and goats from farm 3	p12a	p3, p12b		
Poschiavo Valley	Red deer 6		p14		
Ticino	Red deer 11				d6
Ticino	Red deer 12		d7		
Ticino	Red deer 15		d110		
Ticino	Red deer 17		d10, d111		
Ticino	Red deer 25	d13			
Ticino	Red deer 30	d86	d15, d98	d85	d79, d81, d104
Ticino	Red deer 35		d49		
Ticino	Red deer 37		d18		
Ticino	Red deer 39	d19, d53			
Ticino	Red deer 48		d75	d22	
Ticino	Red deer 141		d27		
Ticino	Red deer 146		d31		
Ticino	Chamois 375	c30			

[a] d, isolate from ticks collected from deer in Ticino; p, isolate from sheep and goats from Poschiavo Valley.

ples. In most of the other 14 cases, DNA extraction was attempted using fully engorged females.

DISCUSSION

The unexpected identification of *B. bigemina* in Switzerland caused concern that this parasite from warmer regions of southern Europe had extended its range. However, this preliminary survey indicates that *B. bigemina* is probably not established in Switzerland as this parasite could not be detected in more than 2,000 ticks collected from domestic and wild ruminants in two regions in southern Switzerland (Poschiavo Valley and Ticino). These locations were chosen as study areas because of their warm climate (Ticino) and because a group of 31 animals, which had been added to the cattle herd that experienced the anaplasmosis outbreak in Chur shortly before the onset of the disease, originated from one of these places (Poschiavo Valley) and were suspected to have introduced the pathogens (8). Furthermore, of the ticks identified during this study, only *H. punctata* has been implicated as a vector for *B. bigemina*, but this was not confirmed in recent experiments (27). Finally, no clinical cases of babesiosis caused by a large *Babesia* sp. have been observed in Switzerland since the outbreak in the cattle herd in Chur occurred (unpublished data). Despite a thorough epidemiological investigation of this outbreak (8) and genetic characterizations of the *B. bigemina* isolate (Hilpertshauser et al., submitted), the origin and the manner of introduction of *B. bigemina* into Switzerland remain unclear.

The spectrum of ticks identified in this study (*I. ricinus*, *D. marginatus*, and *H. punctata*) was the expected spectrum as these species have been described in previous reports from these regions (3). In contrast, the diversity of *Babesia* spp. (*B. divergens*, *B. major*, *Babesia* sp. genotype EU1, new *Babesia* sp. genotype CH1) found in these ticks collected from domestic and wild ruminants was quite surprising. *B. divergens* is known to occur sporadically in the study area in cattle (7), and DNA of this parasite has recently been detected in 2 of 294 *I. ricinus* ticks examined from this region (9). Also, the presence of the zoonotic *Babesia* sp. genotype EU1, which was first identified in two splenectomized patients from Italy and Austria (17), was confirmed in 3 of the 294 ticks examined in that study (9). It has been suggested that *I. ricinus* is a vector for *Babesia* sp. genotype EU1 (13). This suggestion is strongly supported by our findings as we detected this parasite not just in this tick species but, importantly, in *I. ricinus* males, which do not feed, suggesting that there is transstadial transmission of *Babesia* sp. genotype EU1 in *I. ricinus*. PCR analysis of one *H. punctata* tick collected from wild ruminants, which have been identified as hosts of *Babesia* sp. genotype EU1 (13), also revealed the presence of this piroplasm genotype, but whether this tick species acts as a vector or whether *Babesia* sp. genotype EU1 was just taken up by the blood meal without further development in this tick remains to be elucidated. The suggested occurrence of *B. major* in Switzerland (7) was confirmed by molecular analysis by testing DNA extracted from *I. ricinus*, although *H. punctata* is considered a tick vector of *B. major*. The fourth babesial sequence, that of *Babesia* sp. genotype CH1, has not been described before, and this organism is closely related to *B. odocoilei*, which was isolated for the first time from a white-tailed deer in North America and which is transmitted by *Ixodes scapularis* (19, 20). It seems obvious that wild ruminants could act as hosts for the *Babesia* sp. genotype CH1 detected in ticks from Ticino.

Interestingly, more than one tick containing DNA of *Babesia* sp. was collected from four red deer, and all four *Babesia* species or genotypes identified in this study were found in the ticks from a single red deer (red deer 30). The role of wild ruminants as reservoir hosts of these *Babesia* spp. has to be investigated further. In previous surveys, small *Babesia* species have been detected in wild ruminants. A single report described the presence of a small *Babesia* sp. in Switzerland, as revealed by microscopic examination of blood smears of a chamois, and the parasite was considered a bovine piroplasm (6). In several European countries another *Babesia* sp. was found in game animals and named *B. capreoli*, which is morphologically and serologically similar to *B. divergens* (1, 5, 12, 14, 22). A very recently released 18S rRNA gene sequence of an isolate described as *B. capreoli* which was obtained from European roe deer (S. B. Slemanda, A. Chauvin, A. T. Camacho, L. Malandrin, M. L'Hostis, B. L. Herwaldt, and N. J. Pieniazek, unpublished data) (GenBank accession no. AY726009) indeed revealed that this species is not a separate species from *B. divergens* from cattle (Fig. 1).

In addition, *B. divergens* has been identified by molecular means in reindeer in Scotland (21) and in roe and red deer in Slovenia (13).

Taken together, these observations cast doubt on the postulated strict host specificity of bovine *Babesia*. Accordingly, a wide range of hosts has been reported for *B. odocoilei* (19), which has been detected in white-tailed deer, elk, zoo-reared caribou, and reindeer in North America.

Finally, wildlife, especially game animals, is important for maintenance of the tick population (23). Because of changes in land use and farming systems, more interaction between domestic and wild ruminants can be expected, resulting in spillover situations for parasite life cycles (23, 25).

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